Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow

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Wnt signalling has an important role in cell fate determination, tissue patterning and tumorigenesis[1–4]. Secreted antagonists of Wnt include Frizzled (Fz)-related proteins (FRPs)[5–7], Cerberus[8], Wnt inhibitory factor (WIF)[9] and Dickkopf (Dkk)[10,11]. FRPs, Cerberus and WIF have all been shown to act by binding and sequestering Wnt. We report a novel mechanism of Wnt-signalling inhibition by human Dkk-1. Dkk-1 demonstrated no interaction with Wnt but bound a single cell surface site with high affinity (K_D = 0.39 nM). Its receptor was detectable in a complex with a relative molecular mass of 240,000 (M_r 240K) with [^{125}I] Dkk-1 by covalent affinity cross-linking. Wnt signalling through β-catenin is mediated by the Fz receptor[12] and a recently identified low-density-lipoprotein-receptor-related co-receptor, LRP6/Arrow[13–15]. Overproduction of the 200K LRP6 protein, but not of Fz, strikingly increased Dkk-1 binding as well as the amount of the 240K cross-linked complex, which was shown to be composed of Dkk-1 and LRP6. Moreover, Dkk-1 function was completely independent of Fz but LRP6 dramatically interfered with the Dkk-1 inhibition of Wnt signalling. Thus, unlike Wnt antagonists, which exert their effects by molecular mimicry of Fz[5–7] or Wnt sequestration through other mechanisms[8,9], Dkk-1 specifically inhibits canonical Wnt signalling by binding to the LRP6 component of the receptor complex.

Wnt signals through its receptors to inhibit β-catenin phosphorylation by GSK3-β as part of a large cytoplasmic complex that includes Dishevelled (Dvl), casein kinase I, Axin, APC and Frat1 (refs 16,17). Inhibition of β-catenin phosphorylation impairs its degradation, resulting in increased β-catenin translocation to the nucleus and interaction with TCF/LEF transcription factors[18]. To investigate the mechanism of Wnt signalling inhibition by Dkk-1, we investigated its effects on TCF-dependent reporter activity induced in 293T cells by Wnt, β-catenin and several components of the complex required for β-catenin degradation. As shown in Fig. 1a, Wnt and human Fz1 (HFz1) coexpression strongly induced TCF reporter activity, which was inhibited by more than 85% by Dkk-1. Under the same conditions, β-catenin, Dvl, casein kinase I and Frat1 also induced increased reporter activity, which was not significantly affected by Dkk-1. These findings indicated that Dkk-1 inhibition in mammalian cells occurred upstream of the β-catenin degradation complex, consistent with genetic evidence in *Xenopus*[10].

Other Wnt inhibitors have the ability to interact with the ligand, and so we next sought to determine whether Dkk-1 could be detected in a complex with Wnt. As shown in Fig. 1b, Wnt immunoprecipitated with FRP, as has been reported[6,7]. However, there was no detectable interaction between Wnt and Dkk-1 (Fig. 1b), implying that the mechanism of Wnt inhibition by Dkk-1 differed from that of other Wnt antagonists[6,8,9].

To further characterize Dkk-1, we immunoaffinity purified the recombinant Flag-tagged protein using Flag beads (Fig. 2). The protein eluted as a 35–40K species (Fig. 2a) whose biological activity could be measured after purification. Dkk-1 inhibited Wnt induced β-catenin upregulation at subnanomolar concentration

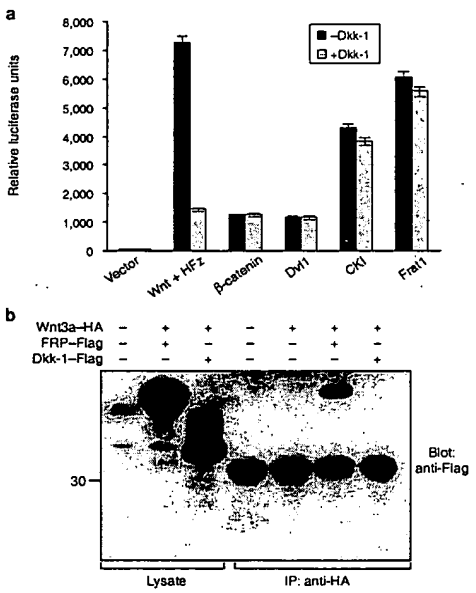

Figure 1 **Analysis of DKK-1 inhibitory function. a**, Dkk-1 inhibits Wnt signalling upstream of the β-catenin degradation complex. 293T cells were co-transfected with DNAs encoding TCF luciferase wild-type or mutant reporters (pGL3-OT, pGL3-OF), 0.1 μg of β-galactosidase reporter and 1 μg of vector or cDNAs for Wnt3a and HFz1, wild-type β-catenin, Dvl1, CKI or Frat1. At 24 h after transfection, Dkk-1 was added at a final concentration of 300 pM and luciferase levels were measured 24 h later. Relative luciferase units (RLU) were normalized for transfection efficiency using β-galactosidase activity and then calculated by subtracting the RLU value obtained with the mutant reporter from that obtained with the wild-type reporter. The results are expressed as mean ± s.d. of two independent experiments performed in duplicate. **b**, Lack of interaction between Dkk-1 and Wnt. 293T cells were transfected with vector control or cDNAs for Wnt3a–HA (haemagglutinin) alone or in combination with either FRP–Flag or Dkk-1–Flag. At 48 h, lysates were analysed by SDS–PAGE, directly or after immunoprecipitation with anti-HA antibody. Immunoblotting was performed with anti-Flag antibody.

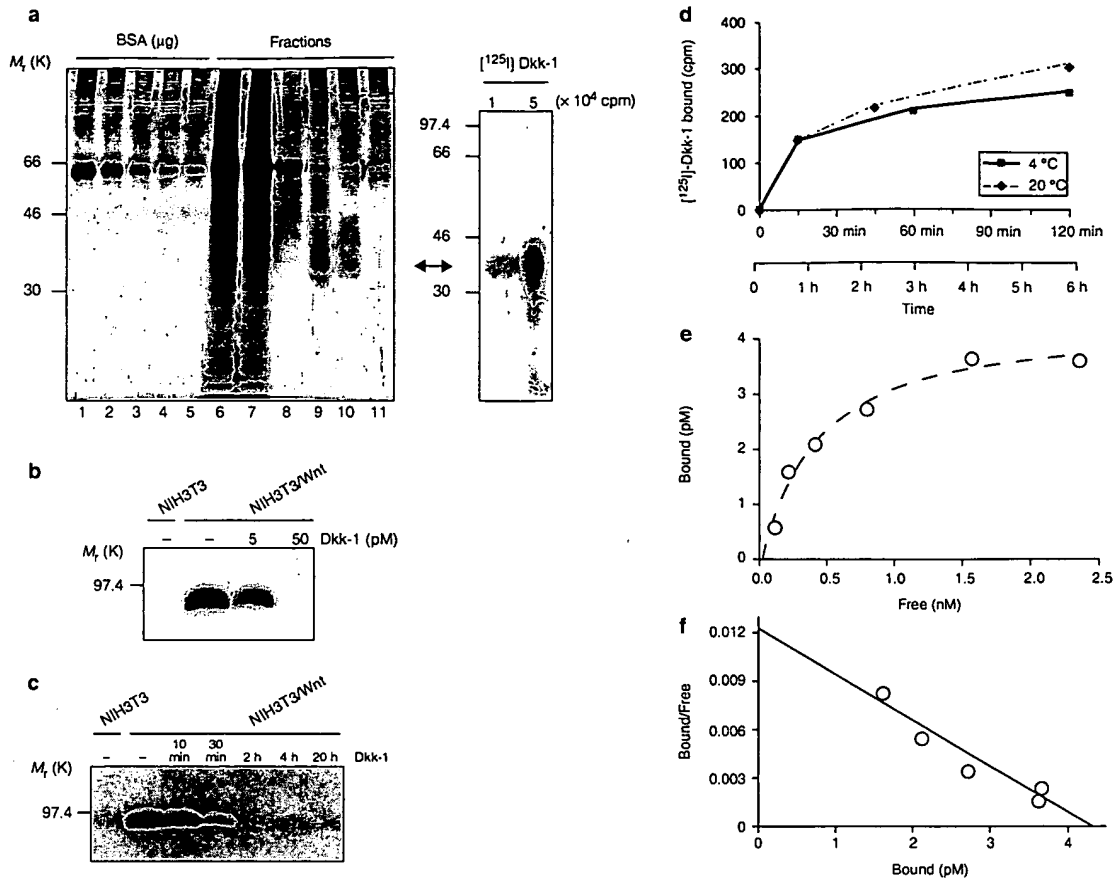

Figure 2 **DKK-1 purification and characterization. a**, Purification and labelling of Dkk-1. Concentrated Dkk-1-conditioned media from Dkk-1-expressing 293T cells was purified through an anti-Flag affinity column. Column fractions were analysed by 10% SDS–PAGE and visualized by silver staining. Dkk-1 eluted as a major 35K species, whose amount was estimated to be around 50 ng (lane 9) by comparison with bovine serum albumin (BSA) run at 200, 100, 50, 20 and 10 ng (lanes 1–5). Starting material (lane 6), flow through (lane 7), column wash (lane 8) and fractions (lanes 9–11) obtained by elution with the Flag peptide are shown. Dkk-1 purification was greater than 10,000-fold by this method (left panel). Dkk-1 (5 μg) was labelled with Na ^{125}I by the chloramine-T method[27] and migrated as a single 35K species by SDS–PAGE (right panel). **b**, Dkk-1 antagonizes Wnt signalling at sub-nanomolar concentration. NIH3T3 cells stably transfected with Wnt2 (ref. 11) were exposed to Dkk-1. After 1 h, cell lysates (1 mg) were obtained and subjected to the GST–E-cadherin assay[26] to detect uncomplexed β-catenin. Dkk-1 inhibition was detected at 5 pM, with complete inhibition at 50 pM. **c**, Kinetics of Dkk-1 inhibition of Wnt signalling. Wnt expressing NIH3T3 cells were incubated for increasing time with Dkk-1 (50 pM), and cell lysates were analysed for uncomplexed β-catenin. Wnt inhibition, detectable within 10 min of Dkk-1 treatment, was complete within 2 h and maintained for at least 20 h. **d**, Time course of Dkk-1 binding to NIH3T3 cells. NIH3T3 cells were incubated with [^{125}I] Dkk-1 (see Methods), at either 20 °C or 4 °C, in the presence of heparin (1 mg ml^{-1}) and in the presence or absence of a 100-fold excess of unlabelled Dkk-1. At different times, the cells were lysed and counted in a γ-radiation counter. Specific binding was calculated by subtracting binding obtained in the presence of a 100-fold excess of unlabelled Dkk-1 from total binding. The results reflect mean values (± s.d.) obtained in two independent experiments performed in duplicate. The shapes of the binding curves were similar at the two temperatures, although the time course was longer at 4 °C (lower axis). **e**, Saturation binding of Dkk-1 on NIH3T3 cells. NIH3T3 cells were incubated with increasing amounts of [^{125}I] Dkk-1 in the presence or absence of a 100-fold excess of unlabelled Dkk-1. Cells were lysed and counted as described in the Methods. Values are the mean of duplicate samples. The results were similar when performed at 20 °C or at 4 °C. **f**, A Scatchard plot of the data for [^{125}I] Dkk-1 binding to NIH3T3 cells (**e**). The vertical axis represents the ratio of bound to free [^{125}I] Dkk-1; the horizontal axis represents the concentration of bound [^{125}I] Dkk-1. The data were analysed by SIGMA PLOT and fitted to a binary-binding model. The K_D was 0.39 nM and there were 2.5×10^3–5.0×10^3 binding sites per cell.

(Fig. 2b), with a marked reduction in β-catenin levels observed within 30 min (Fig. 2c). These findings suggested that Dkk-1 action did not require transcriptional events.

In an effort to characterize its cell surface receptor, we ^{125}I-labelled purified Dkk-1–Flag (Fig. 2a), which demonstrated specific binding to NIH3T3 cells in a time-dependent (Fig. 2d) as well as concentration-dependent manner (Fig. 2e). Scatchard analysis (Fig. 2f) revealed a single receptor population consisting of 2.5×10^3–5.0×10^3 sites per cell with a K_D of 0.39 nM.

We next performed covalent affinity cross-linking of [^{125}I] Dkk-1–Flag to NIH3T3 cells using a membrane-impermeable cross-linker (BS3). SDS–PAGE of cell lysates revealed a major complex of 230–240K (Fig. 3a) whose specificity was demonstrated by competition with an excess of unlabeled Dkk-1. The recent identification of LRP6 as a component of the Wnt receptor complex and know-ledge of its predicted size (200K)[15] suggested that this cross-linked complex might be composed of Dkk-1 and LRP6. To test this possibility, we investigated the effects of transfection with LRP6 or HFz1 on [^{125}I] Dkk-1 binding to 293T cells. Although [^{125}I] Dkk-1 binding was not detectably increased by HFz1 overexpression, LRP6 transfection increased Dkk-1 binding almost tenfold (Fig. 3b). Notably, overexpression of Wnt resulted in no detectable increase in Dkk-1 binding to cells, further indicating the lack of interaction between Wnt and Dkk-1. LRP5 is a closely related homologue of LRP6 and has been reported to enhance canonical Wnt signalling, although not as efficiently as LRP6 (ref. 15). LRP5

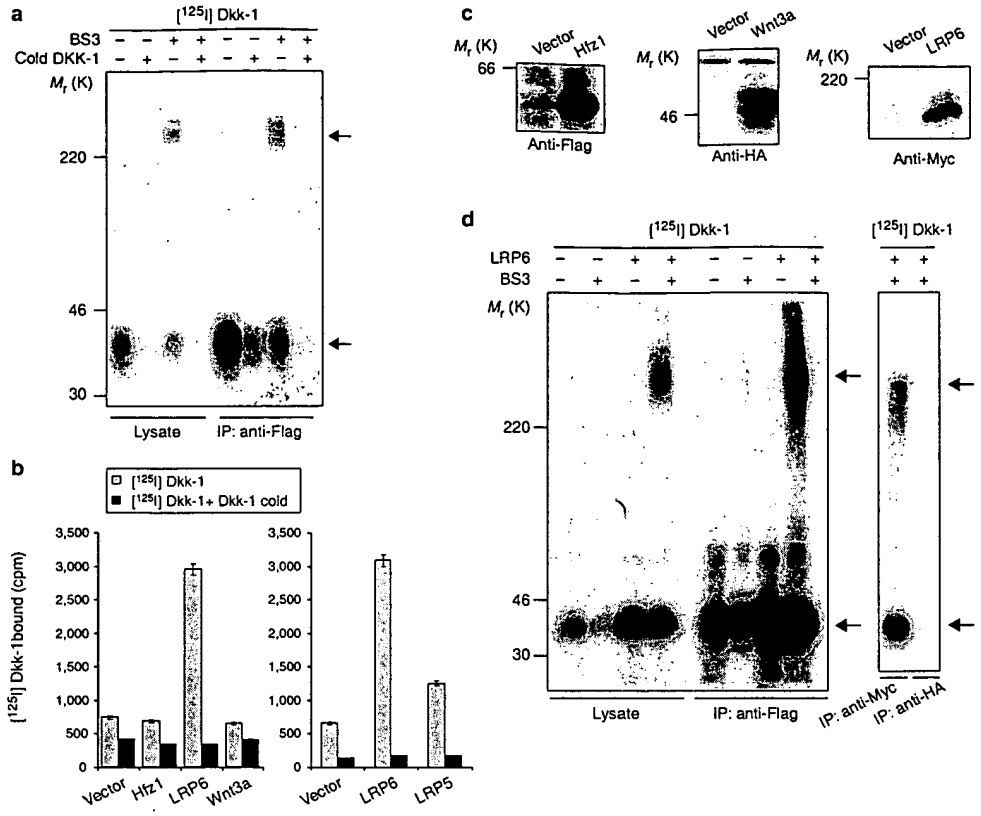

Figure 3 **DKK-1 binds specifically to LRP5/6.** **a**, Covalent affinity cross-linking of [125I] Dkk-1. NIH3T3 cells were incubated with [125I] Dkk-1–Flag as described in the Methods and Fig. 2d and treated with BS3, and cell lysates were analysed by 8% SDS–PAGE directly or after immmunoprecipitation with anti-Flag antibody. Arrows indicate the 35K [125I] Dkk-1 and the 240K cross-linked complex. Specificity of Dkk-1 binding and of the 240K complex was shown by reduction of the signals in the presence of a 100-fold excess of unlabelled Dkk-1 as competitor (cold Dkk-1). **b**, Overexpression of *LRP6* increases specific binding of [125I] Dkk-1. 293T cells were transfected with 1 μg of vector, *HFz1*, *LRP6* or *Wnt3a* (left panel) and 1 μg of vector, *LRP6* or *LRP5* (right panel). Binding was performed as for Fig. 2d and Methods, in the absence or presence of a 100-fold excess of cold Dkk-1. **c**, Expression of tagged *HFz1*, *Wnt3a* and *LRP6* in 293T cells. Immunoblot analysis was performed as described[24] using anti-Flag, anti-HA or anti-Myc antibodies. Molecular weight markers are indicated. **d**, [125I] Dkk-1 covalent affinity cross-linking on *LRP6*-expressing 293T cells. 293T cells transfected with either vector control or *LRP6–Myc* were incubated with [125I] Dkk-1–Flag, treated with BS3 and analysed by stacking SDS–PAGE (bottom 10%, top 6%). Lysates were analysed directly or after immunoprecipitation with either anti-Flag beads (more efficient than the anti-Flag antibody; Fig. 3a) anti-Myc antibody or anti-HA antibody. Arrows indicate [125I] Dkk-1 (35K) and the 240K [125I] Dkk-1 complex.

also increased Dkk-1 binding although to a lesser extent than LRP6 (Fig. 3b). Figure 3c shows that each of the tagged molecules was expressed efficiently.

To establish evidence of direct interaction between Dkk-1 and LRP6, we performed cross-linking experiments on 293T cells overexpressing LRP6–Myc. As shown in Fig. 3d, LRP6–Myc strikingly increased the signal of the cross-linked complex detected. Moreover, immunoprecipitation with anti-Flag beads revealed that the complex was indistinguishable in size from the endogenous 240K complex. This size implied a binary complex of Dkk-1 and LRP6–Myc (35K and 200K, respectively) that was directly demonstrated by the ability of the anti-Myc antibody, which immunoprecipitated LRP6–Myc (data not shown), to immunoprecipitate the 240K complex (Fig. 3d). The 35K Dkk-1 species immunoprecipitated must reflect [125I] Dkk-1 bound but not cross-linked to LRP6, because neither the 240K complex nor [125I] Dkk-1 was immunoprecipitated by an unrelated antibody (Fig. 3d).

Dkk-1 interaction with LRP6 might initiate an intracellular signal responsible for Wnt inhibition at a level upstream of the β-catenin degradation complex. If so, increased LRP6 expression would be expected to increase Wnt sensitivity to Dkk-1 by providing more inhibitory signalling receptors. As shown in Fig. 4, inhibition of TCF reporter activity induced in 293T cells by Wnt alone was observed at Dkk-1 concentrations as low as 30 pM and was half maximal in the range 0.3–1.0 nM. The somewhat lower Dkk-1 sensitivity of Wnt-transfected 293T cells compared with Wnt-expressing NIH3T3 cells (Fig. 2b) might reflect higher levels of Wnt expression by 293T cells (data not shown) or cell-specific differences. Notably, despite the fact that HFz1 co-transfection with Wnt stimulated reporter activity nine- to tenfold more than Wnt alone (Fig. 4, inset), HFz1 overexpression had no effect on Wnt signalling inhibition by Dkk-1 (Fig. 4). By contrast, co-transfection of LRP6 and Wnt, which increased TCF reporter activity around fourfold over that of Wnt alone (Fig. 4, inset), increased the concentration of Dkk-1 required for comparable inhibition more than 100-fold. These results exclude a mechanism by which Dkk-1 transmits an inhibitory intracellular signal through LRP6. Instead, our findings imply that Dkk-1 binding to LRP6 interferes with the functional interaction between Wnt and its receptor complex.

Wnt binds to LRP6 as well as to Fz and is required for the two co-receptors to form a complex[15]. Moreover, the cytoplasmic domains of both LRP6 and Fz seem to be required for Wnt signalling[15,19]. The ability of Dkk-1 functionally to inhibit Wnt signalling was shown to be independent of Fz but to be influenced by LRP6, whose increased expression dramatically interfered with Dkk-1 activity. In *Drosophila*, the LRP6 homologue, Arrow, has been shown to be involved in Wnt–β-catenin signalling but not in Wnt-induced planar polarity[13]. Our present demonstration that

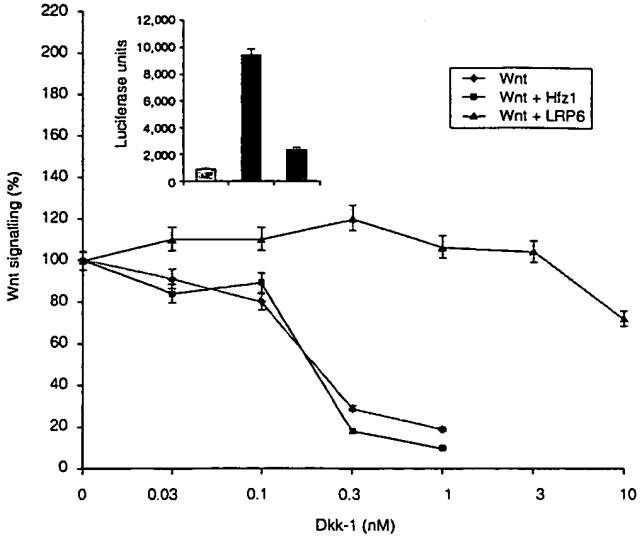

Figure 4 **Effects of LRP6 on Dkk-1 functional inhibition of Wnt signalling.** 293T cells were transfected as described previously[24] using vector, *Wnt3a*, *Wnt3a* and *HFz1*, or *Wnt3a* and *LRP6* in the presence of the wild-type or mutant TCF/Luc reporters and the β-galactosidase reporter. At 24 h after transfection, Dkk-1 was added, and luciferase levels were measured 24 h later. Results were obtained as for Fig. 1a and expressed as mean ± s.d. of two independent experiments performed in duplicate. Luciferase levels obtained in the absence of Dkk-1 are shown (inset) and the effect of increasing Dkk-1 concentrations on these respective levels are indicated as a percentage.

Dkk-1 interacts in a bimolecular complex with LRP6/Arrow establishes Dkk-1 as a specific antagonist of canonical Wnt signalling.

Most proteins that antagonize ligand–receptor interactions act through molecular mimicry to inhibit ligand binding to the receptor competitively. Examples include alternative splice products yielding secreted ligand-binding domains of receptors[20] or ligand antagonists[21] as well as related antagonists that are products of distinct genes[20,22]. Among Wnt antagonists, only FRP has a structure that closely resembles the ligand-binding domain of the Fz receptor and conforms to this model. FRP binds both to Wnt and Fz, potentially interfering with Wnt–Fz interactions at both levels[6,7]. Dkk-1, Cerberus and WIF are structurally distinct from one ano-ther as well as from Wnt and Fz. Nonetheless, Cerberus and WIF bind to, and presumably sequester, Wnt[8,9]. Our present findings establish that Dkk-1 acts by a mechanism that does not involve interaction with Wnt but is mediated by its direct interaction with LRP6. Notably, three of the four classes of Wnt signalling inhibitors, including Dkk, FRP and Cerberus, have no homologues in *Drosophila* (genome database analysis, not shown). All of these findings attest to the critical importance of the evolution of diffe-rent classes of Wnt inhibitors in modulating the spatial and temporal pattern of Wnt activity involved in vertebrate development. □

Methods

Constructs.

To construct pcDNA3–Dkk-1–Flag, the Dkk-1 coding region was PCR amplified and inserted into pcDNA3, upstream of the Flag M2 epitope tag. pSK–β-catenin–Myc, provided by W. Birchmeier[23], was subcloned into pcDNA3. Human *Dvl1* cDNA was amplified by PCR from human breast epithelial cell cDNA. Mouse *CKI* and *Frat1* cDNAs were amplified by PCR from an E11d mouse embryonic cDNA pool. PCR products were cloned into the expression vector pCCBS-puro containing an amino-terminal Myc tag. The plasmids pBabe–Wnt2HFc, pCEV29–HFz1, pLNC–Wnt3a (ref. 24) and pcDNA3–FRP–Flag (ref. 7), and the *LRP5* and *LRP6* cDNAs (refs 15, 25) have been described. The 3′ region of LRP6 c-DNA was PCR amplified with a 5′ primer from a unique internal *Eco*RI site and a 3′ primer containing a *myc*-Tag in frame with the end of the coding sequence. It was then ligated to the LRP6 5′ region through this *Eco*RI site, and cloned into pcDNA3.

Luciferase assays.

The TCF luciferase constructs pGL3-OT and pGL3-OF, provided by B. Vogelstein (John Hopkins Oncology Center, Baltimore, Maryland, USA), were used for transcriptional reporter assays as reported[24].

GST–E-cadherin binding assay.

The GST–E-cadherin assay was performed as described[26]. β-Catenin was detected using a monoclonal antibody (Transduction Laboratories).

Cell culture and transfection.

293T cells were transiently transfected using Fugene (Boehringer Manheim) as described[24]. NIH 3T3 cells stably expressing Wnt2 have been reported[11].

Protein purification, iodination and binding analysis.

Serum-free conditioned medium was collected from *Dkk-1–Flag* transfected 293T cells and concentrated (tenfold) using centrifugal filters containing membranes with a 10K size cut-off. Purification of the Flag-tagged Dkk-1 was performed by anti-Flag M2 affinity chromatography followed by competitive elution using the Flag peptide according to the manufacturers instructions (Sigma). The eluted Dkk-1 was analysed by SDS–PAGE followed by silver staining. Dkk-1 labelling was performed with [^{125}I] sodium iodide by the chloramine-T method[27].

For the binding assay, cells in 24-well plates were washed twice in binding buffer (BB; serum-free growth medium, 0.1% bovine serum albimun, 25 mM Hepes pH 7.4) and incubated for varying times either at 20 °C or 4 °C in BB containing [^{125}I] Dkk-1 (50,000 cpm per well) in the absence or presence of a 100-fold excess of unlabelled ligand. Dkk-1 is an heparin-binding protein[11] and specific binding was found to be optimized in the presence of heparin (1 μg ml^{-1}). Cells were washed twice in PBS, lysed in 0.5% SDS and counted in a γ-radiation counter. For saturation binding and Scatchard analysis, samples were incubated with increasing concentrations of [^{125}I] Dkk-1 in the absence or presence of a 100-fold excess of unlabelled Dkk-1 and processed as above. Estimates of receptor affinity and total binding capacity were made using SIGMA PLOT software.

Covalent-affinity cross-linking, immunoprecipitation and immunoblotting

For cross-linking experiments, cells were incubated with the [^{125}I] Dkk-1 (3×10^6 cpm per 100 mm plate) as described above and then treated for 1 h with bis-sulfosuccinimidyl-suberate (BS3), a membrane-impermeable cross-linker (Pierce). The reaction was stopped by addition of 20 mM Tris (pH 7.5). Cells were washed twice in PBS and solubilized in lysing buffer (10 mM sodium phosphate pH 7, 150 mM NaCl, 1% NP40) with protease inhibitors. After SDS–PAGE analysis, gels were fixed, dried and exposed. Immunoprecipitation was performed using anti-Myc antibody, anti-haemagglutinin (anti-HA) antibody, anti-Flag antibody or the anti-Flag affinity beads. Co-immunoprecipitation and immunoblotting were performed as previously described[7].

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